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Determination of Free and Total Available Ferulic Acid in Different Types of Chinese Angelica by High Performance Liquid Chromatography

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ABSTRACT

Free and conjugated forms of ferulic acid (FA) are generally available in higher plant taxa such as Chinese Angelica (**CA**, the roots of *Angelica sinensis* (Oliv.) Diels). These various forms of FA were found readily inter-convertible and the extractable level of each depended on solvent properties and acidity. Extraction efficiency using various pH solutions namely, water, 70% methanol, methanol-formic acid (95:5) and methanol-2% $NaHCO_3$ in water (95:5) was compared. Extractable FA were found varying in samples under neutral solvents extraction, whilst relatively consistent for slightly acidic and alkali solvents which were therefore chosen as the optimal media to extract and determine the reproducible levels of free and total available FA. An accurate and rapid high performance liquid chromatographic (HPLC) analysis was conducted using an Alltima C18 column (5 μm, 4.6 mm i.d. × 250 mm) with a guard column (C_{18}, 5 μm, 4.6 mm i.d. × 7.5 mm) at 30°C, eluted with a mixture of 1.0% acetic acid and acetonitrile in a gradient program at a flow rate of 1.0 mL/min and detected at 320 nm. Altogether 20 different types of **CA** samples including whole root, root head, rootlets, whole root slice, Angelica processed by Chinese yellow wine, and charred Angelica were quantified for free and total available FA. Total available FA was found more abundant than free counterpart with an average ratio of 3.15 (n = 20) in the range of 1.29 to 8.23 for these **CA** samples. The extraction protocol was proven reliable to quantitatively convert all conjugated FA into its free forms and thereby accurately determined by HPLC method for quality assessment.

Key words: ferulic acid, *Angelica sinensis*, quantitative analysis, HPLC

INTRODUCTION

Ferulic acid (FA) is a small molecular weight chemical compound available in Chinese Angelica (**CA**, the roots of *Angelica sinensis* (Oliv.) Diels) and higher plant taxa[(1-3)]. Pharmacological studies showed that FA possessed several actions such as inhibiting platelet aggregation, increasing coronary blood flow, antiarrhythmia, immunostimulation, and antioxidant activity, etc.[(4-9)]. Its water-soluble form (sodium ferulate) is available in various dosage forms of drugs, e.g., sodium ferulate tablet, sodium ferulate injection and sodium ferulate powder[(10)]. Therefore, FA is generally selected as the chemical marker to assess the quality of **CA** and Chinese Lovage (the rhizome and root of *Ligusticum sinensis* Oliv. and *L. jeholense* Nakai et Kitag.) as recorded in the Pharmacopoeia of the People's Republic of China[(11,12)] and the China Chinese Medicine Pharmacopoeia[(13)].

However, FA usually coexists with its conjugated forms, e.g. coniferyl ferulate and phenethyl ferulate, etc[(1,14-16)]. Some conjugated compounds are readily convertible in different extents during sample extraction, thereby resulting in varied FA content (Figure 1)[(17)]. In literatures, FA was mostly extracted and quantified in herbs using neutral solvents (methanol, aqueous methanol, ethanol, aqueous ethanol, mixture of diethyl ether-methanol and water) and weak acidic solvents (5% formic acid or 1-5% acetic acid in methanol)[(2,3,11-13,18-22)]. From these works, it was observed that FA content varied significantly among a particular herb samples. Despite the variation in natural abundance among samples, the uncontrollable conversion among various forms during

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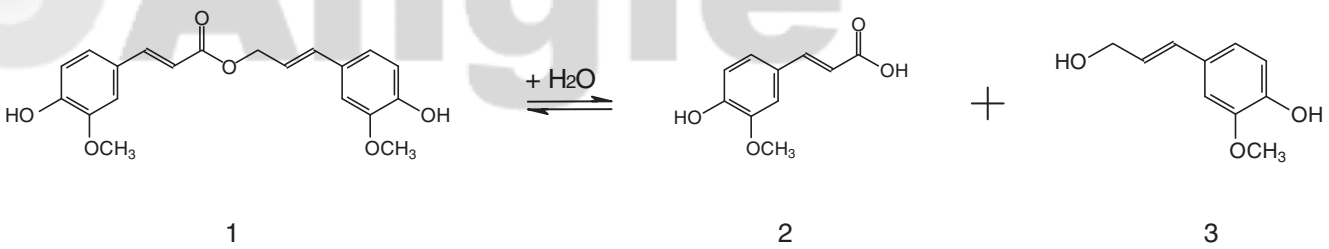

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Figure 1. Chemical equilibrium of (1) coniferyl ferulate with (2) ferulic acid and (3) coniferyl alcohol.

extraction was likely to be the crucial source of variation. Owing to the acidity of FA, conversion of conjugated forms was suppressed in a slightly acidic medium resulting in less FA production. Note that this conversion occurred more readily in alkaline or neutral media leading to more detectable FA content. Therefore, fluctuation in the quantifiable amount of FA was influenced by samples extracted under various pH conditions. In light of this, optimized and controllable pH conditions for extraction were critical factors to retrieve realistic amount of FA in herbs for quality assessment. However, little attention had been devoted to the variability of FA under different extraction pH in current practices of FA determination in plant materials. The reported data was neither the natural abundance (free FA) nor the total available amount (total available FA) in herbs.

Our previous study researched the variation of extractable FA under different conditions[23]. Considered the latest official analytical methods stipulated in the Pharmacopoeias in mainland China, Taiwan and in recent literatures, the present work is focused on the optimization of extraction condition using various pH solvents to quantitatively isolate free FA and to investigate the available amount of FA in **CA** contributing to the therapeutic effect in Chinese medicinal practice by high-performance liquid chromatography (HPLC)-diode array detection (DAD)-mass spectrometry (MS) techniques. An accurate and rapid HPLC method was therefore developed to determine the reliable FA content in **CA** samples. Altogether 20 **CA** samples including whole root, root head, rootlets, whole root slice, Angelica processed with Chinese yellow wine, and charred Angelica were quantified for the amount of free and total available FA. These findings provided scientific basis to determine the accurate, reliable and comparable amount of FA in **CA** materials. The developed analytical procedure is reproducible, rapid and suitable for the quantitative analysis of FA in large number of samples.

MATERIALS AND METHODS

I. *Materials*

Twenty **CA** samples were collected from mainland China and Hong Kong (Table 1). These samples were authenticated by Prof. Zhong-Zhen Zhao (School of Chinese Medicine, Hong Kong Baptist University, Hong Kong) and voucher specimens were preserved in this institution.

II. *Chemicals and Reagents*

FA was purchased from the Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). Methanol (analytical reagent grade) and acetonitrile (HPLC grade) were purchased from Labscan (Bangkok, Thailand). Analytical graded formic acid, glacial acetic acid and sodium hydrogen carbonate ($NaHCO_3$) were obtained from Uni-chem (Warsaw, Poland). Deionized water was prepared by a Milli-Q water system (Millipore, MA, USA).

III. *Instruments and Conditions*

HPLC was conducted using an Agilent 1100 series HPLC-DAD system (Agilent, CA, USA). An Applied Biosystems/PE-SCIEX API 365 LC-MS-MS system with atmospheric pressure chemical ionisation (APCI) source (Applied Biosystems, CA, USA) was used for MS analysis. A Branson 5210E-MTH ultrasonic processor (Branso, CT, USA. Output: 135 watts, 42 KHz ± 6%) was used for sample extraction. The pH value was measured by a Sartorius Basic Meter PB-10 (Sartorious AG, Goettingen, Germany).

The analytical column was an Alltima C18 column (5 μm, 4.6 mm i.d. × 250 mm) equipped with a guard column (C_{18}, 5 μm, 4.6 mm i.d. × 7.5 mm). The mobile phase consisted of 1.0% acetic acid in water (**A**) and acetonitrile (**B**) using a gradient program of 19% B in 0-18 min, 19-100% **B** in 18-60 min. Detection wavelength was set at 320 nm and column temperature was maintained at 30°C. Flow rate was set at 1.0 mL/min. An aliquot of 10 μL standard or sample solution was injected. Online UV spectra were acquired from 200 to 400 nm. The APCI-MS spectra were acquired in both positive and negative ion modes.

IV. *Preparation of Standards Solution*

FA standard stock solution was prepared at a concentration of 200 mg/L by transferring 5.0 mg of FA standard into a 25-mL amber volumetric flask and made up to volume with methanol. This stock solution was then dilut-

Table 1. Free and total available ferulic acid content in different types of Chinese Angelica samples

No.	Sample type	Source (Collection year)	Free ferulic acid[b]	Total available ferulic acid[b]	Ratio[c]
1	Whole root	Hong Kong (2002)	0.387 ± 0.006	1.219 ± 0.016	3.15
2	Whole root	Hong Kong (2006)	0.202 ± 0.003	1.135 ± 0.009	5.62
3	Whole root	Pingwu, Sichuan, China (2003)	0.177 ± 0.001	1.056 ± 0.006	5.97
4	Whole root	Guangzhou,China (2005)	0.111 ± 0.001	0.914 ± 0.002	8.23
5	Whole root	Chengdu, China (2005)	0.383 ± 0.001	0.991 ± 0.006	2.59
6	Root head	Guangzhou, China (2005)	0.177 ± 0.005	0.521 ± 0.013	2.94
7	Root head	Xiamen, China (2006)	0.308 ± 0.004	0.658 ± 0.002	2.14
8	Root head	Hong Kong (2006)	0.143 ± 0.004	0.887 ± 0.012	6.20
9	Rootlets	Xiamen, Fujian, China (2006)	0.430 ± 0.004	1.030 ± 0.004	2.40
10	Rootlets	Hong Kong (2005)	0.369 ± 0.011	0.858 ± 0.002	2.33
11	Rootlets	Hong Kong (2006)	0.164 ± 0.006	0.940 ± 0.009	5.73
12	Whole root slice	Chengdu, China (2005)	0.453 ± 0.017	0.763 ± 0.018	1.68
13	Whole root slice	Guangzhou, China (2005)	0.193 ± 0.004	0.439 ± 0.002	2.27
14	Whole root slice	Hong Kong (2006)	0.339 ± 0.003	0.557 ± 0.012	1.64
15	Wined Angelica[a]	Chengdu, China (2005)	0.424 ± 0.007	0.703 ± 0.019	1.66
16	Wined Angelica	Shenzhen, China (2006)	0.262 ± 0.001	0.431 ± 0.008	1.65
17	Wined Angelica	Anguo, Hebei, China (2006)	0.357 ± 0.005	0.776 ± 0.032	2.17
18	Charred Angelica	Hong Kong (2005)	0.014 ± 0.0004	0.018 ± 0.001	1.29
19	Charred Angelica	Shenzhen, China (2006)	0.035 ± 0.001	0.057 ± 0.001	1.63
20	Charred Angelica	Xiamen, China (2006)	0.348 ± 0.004	0.621 ± 0.004	1.78

[a]Wined Angelica was Angelica processed by Chinese yellow wine.
[b]The value was mean ± SD (n = 4), mg/g.
[c]The ratio of the amount of total available to free ferulic acid in sample.

ed to obtain a set of calibration solutions in the concentration range of 1-25 mg/L comprising 7 concentration levels.

V. *Preparation of Sample Solution*

Representative dried herb samples were cut into smaller pieces, ground into powder and passed through a 45-mesh (opening 355 μm) sieve (Cole-Parmer, USA). For the assay of free FA, a powder sample (0.5 g) was accurately weighed and transferred into a 60-mL amber vial and a quantity of 25 mL of methanol-formic acid (95:5) was added and sonicated for 60 min. The extract was normalized to 25 mL using extraction solvent and filtered through a 0.2 μm membrane filter for HPLC analysis. Sample duplicates were prepared. For the assay of total available FA, sample solutions were prepared following similar procedures as the above except using methanol-2% $NaHCO_3$ in water (95:5) and sonicated for 100 min.

RESULTS AND DISSCUSSION

I. *Identification of Ferulic Acid and Coniferyl Ferulate*

Peak intensity of FA and coniferyl ferulate (CF) was found to be remarkably different in the HPLC chromatograms of these extracts and therefore assigned for comparative purpose. FA was identified by comparing reference standard with respect to retention time, UV and APCI-MS data. In the APCI-MS spectra of FA, a strong deprotonated molecular ion $[M - H]^-$ at 193 *m/z* together with a negative fragment ion at 179 *m/z* were observed. Absorption maximum in UV spectrum was observed to be 323 nm in the acidic mobile phase (pH 2.90). Due to the unavailability of a reference compound, tentative identification of CF was based on the on-line HPLC-APCI-MS and UV data and compared with those reported in the literatures[14,24,25]. In APCI-MS negative mode, the deprotonated molecular ion $[M - H]^-$ was observed at 355 *m/z*. A strong response of negative fragment ion was found at 193 *m/z* and assigned as the FA moiety of CF. In APCI-MS positive mode, the strongest positive fragment ion was located at 163 *m/z* and assigned as the coniferyl alcohol moiety of CF. Its maximum absorption in UV spectra was shown at 318 nm.

II. *Evaluation of the Extraction Efficiency of Various pH Solvents*

The four solvent systems included water (pH 6.64),

70% methanol (pH 6.14), methanol-formic acid (95:5, pH 3.23), and methanol-2% $NaHCO_3$ in water (95:5, pH 10.5). The available amount of FA in **CA** contributing to the therapeutic effect in medicinal decoction was carefully investigated. Quantification of FA was conducted on standard calibration curve using certified reference chemical standards. As CF standard was unavailable, specific peak area (peak area / sample weight, mAU s/g) was used for assay purpose.

(I) Water: As a general practice, the decoctions of Chinese medicinal materials are always prepared in water. Aqueous extraction was also commonly used for the assay of FA in herbs by capillary electrophoresis[21,22]. Therefore, water was chosen as extraction solvent to investigate the available amount of FA contributing to the therapeutic effect in Chinese medicines. Two tests were conducted using various extraction procedures. Sample powder was first extracted in water under reflux for 60 min. This aqueous extract was centrifuged and the supernatant was evaporated to dryness under vacuum. This residue was then re-dissolved in methanol for HPLC analysis. The 0.815 mg/g of FA was determined, whereas the peak corresponding to CF was not observed (Figure 2a; Table 2, number 1), possibly due to the fact that CF was sparingly soluble in water[26]. Therefore, another test was designed in which the aqueous extract and the material residue were evaporated to dryness together. This residue was then re-dissolved in methanol and subjected to HPLC analysis. Any CF in the material residue could be thereby dissolved and detected,

Table 2. Comparison on ferulic acids using various pH extraction methods

No.	Solvent[a]	Method	Extraction time (min)	Storage duration before injection	Ferulic acid (mg/g)	Coniferyl ferulate[b]
1	A	Reflux	60		0.815	ND
2	A	Reflux	60		0.790	ND
3	B	Reflux	15		0.543	868.7
4	B	Reflux	30		0.737	188.9
5	B	Reflux	45		0.758	168.9
6	B	Reflux	60		0.775	70.4
7	B	Reflux	90		0.784	11.5
8	B	Reflux	120		0.795	ND
9	B	Reflux	150		0.789	ND
10	B	Reflux	30	1.5 hr	0.737	188.9
11	B	Reflux	30	3.0 hr	0.752	186.6
12	B	Reflux	30	9.5 hr	0.755	167.8
13	B	Reflux	30	25.4 hr	0.766	144.7
14	B	Reflux	30	38.8 hr	0.767	124.4
15	B	Reflux	30	73.4 hr	0.782	99.1
16	C	Sonication	30		0.135	2012.3
17	C	Sonication	45		0.135	2006.6
18	C	Sonication	60		0.135	2009.4
19	C	Sonication	90		0.139	2080.2
20	D	Sonication	60	5 min	0.634	444.4
21	D	Sonication	60	102 min	0.751	90.0
22	D	Sonication	60	198 min	0.794	18.8
23	D	Sonication	60	390 min	0.786	ND
24	D	Sonication	90	6 min	0.788	ND
25	D	Sonication	30	10 hr	0.807	ND
26	D	Sonication	45	10 hr	0.806	ND
27	D	Sonication	60	10 hr	0.806	ND
28	D	Sonication	80	10 hr	0.811	ND
29	D	Sonication	100	10 hr	0.796	ND

[a]A, B, C and D were water, 70% methanol, methanol-formic acid (95:5) and methanol-2% $NaHCO_3$ in water (95:5), respectively.
[b]Specific peak area. The value was the ratio of peak area to sample weight, mAU s/g. ND means not detected.

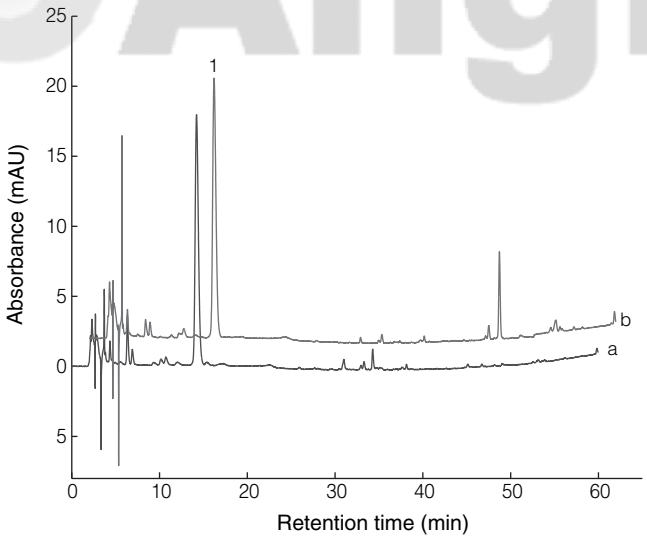

Figure 2. Chromatograms of the root of *Angelica sinensis* (Oliv.) Diels extracted in water under (a) refluxed for 60 min with extract evaporated and re-dissolved in methanol for HPLC analysis; (b) refluxed for 60 min with both extract and material residue evaporated together and re-dissolved in methanol for HPLC analysis. (1) Ferulic acid.

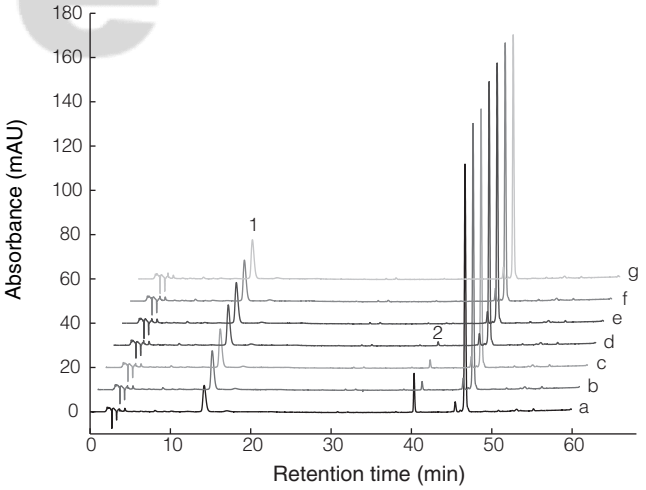

Figure 3. Chromatograms of the root of *Angelica sinensis* (Oliv.) Diels extracted with 70% methanol under refluxed for (a) 15 min; (b) 30 min; (c) 45 min; (d) 60 min; (e) 90 min; (f)120 min and (g) 150 min, respectively. (1) Ferulic acid; (2) Coniferyl ferulate.

but no CF peak was observed (Figure 2b; Table 2, number 2). This observation indicated that solubility of CF was not the cause of its absence in aqueous extract. Indeed, CF was completely hydrolyzed into FA upon prolonged reflux in water where the aqueous extract became weakly acidic (pH 4.68). This result agreed with the report that CF was partially hydrolyzed in water at 90°C for 30 min[26]. The average FA in the two tests was estimated as 0.802 ± 0.018 mg/g (mean ± SD, n = 2), which was suggested as the available amount of FA contributing to the therapeutic effect in Chinese medicine practice. This data was therefore used as a reference of the available FA in this herbal sample for subsequent comparative studies. However, from a technical viewpoint, filtering the aqueous extract for HPLC analysis was fairly difficult and needed prolong evaporation, making the assay using water extraction tedious.

(II) 70% methanol: The Pharmacopoeia of the People's Republic of China (2005 edition) adopted 70% methanol extraction under reflux for 30 min as the official method for FA assay in **CA**[11]. However, this extraction method was not reproducible based on our previous researches. In the current study, sample powder was extracted with 70% methanol under reflux for 15, 30, 45, 60, 90, 120 and 150 min. Peak abundances of FA and CF were observed to be different with regard to extraction duration (Figure 3). The amount of FA was found to increase progressively in the expense of CF over the extended extraction period (Table 2, numbers 3-9). This result indicated that CF was liable to hydrolyze into FA in this solvent, and completely converted over 2 hr of extraction. The amount of FA under reflux for 2 hr and 2.5 hr was in agreement with that of FA extracted in water. Therefore, in order to achieve a complete extraction and reliable result of FA, the sample should be refluxed for more than 2 hr. This finding indicated that extraction method recorded in the Pharmacopoeia of the People's Republic of China (2005 edition) was not optimized and likely resulted in a variable amount of extractable FA.

The standing stabilities of FA and CF obtained from 30-min extraction were also investigated under various storage durations. FA and CF contents were determined in the original extract after standing for 1.5, 3.0, 9.5, 25.4, 38.8 and 73.4 hr. The peak area of FA increased by 6.42% whilst that of CF reduced by 47.5% upon 3 days of standing (Table 2, numbers 10-15). This finding indicated that the variation of FA content in this extract was considered small with a RSD of 2.03% (n = 6), which revealed that the conversion of conjugated compounds into FA mainly occurred during extraction in this neutral medium.

(III) Methanol-formic acid (95:5): This weakly acidic medium was recently adopted as extraction solvent for the determination of FA in herbs[19,20]. Its extraction efficiency was further investigated for the determination of free FA in natural abundance. The sample powders were respectively sonicated for 30, 45, 60 and 90 min, respectively. In general, their chromatographic patterns were found to be consistent (Figure 4). The FA content (0.136 ± 0.002 mg/g, n = 4) and the specific peak area of CF (2026.99 ± 35.54 mAU s/g, n = 4) in these samples were found to be similar with RSD of 1.47% and 1.75% (n = 4), respectively (Table 2, numbers 16-19). It indicated that the content variations of FA and CF were minimized in this acidic medium despite prolonged extraction dura-

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tion from 30 to 90 min. It demonstrated that hydrolysis of CF was suppressed in this weakly acidic medium resulting in conjugated forms as the principal component in the extracts. The determined amount of FA was considered as the natural abundance in free form in herb. Moreover, the amount of FA extracted by sonication for 30 min was found to be similar to the one extracted up to 90 min, showing that free FA in botanical sample was easily extracted and determined with this medium. In consideration of the possible variation in the nature of herb samples, a 60-min extraction was proposed as the optimal sonication time using acidic medium in subsequent studies.

(IV) Methanol-2% $NaHCO_3$ in water (95:5): Normally, both FA and its conjugated compounds in plant materials could be extracted in methanol. However, the latter was likely to be converted into FA during sample extraction[17]. Owing to the acidity of FA, an alkaline medium was presumed to facilitate this conversion as FA would be neutralized into its salt form; and this ionized form could be converted into FA again in an acidic mobile phase during HPLC analysis. Therefore, methanol-2% $NaHCO_3$ in water (95:5) was chosen as the extraction solvent to investigate the form of CF extractable from herbs and the conversion speed of CF into FA in extracts. Three tests were conducted as follows:

Sample powder was first sonicated for 60 min and analyzed upon standing for 5, 102, 198 and 390 min, respectively (Figure 5 a-d; Table 2, numbers 20-23). FA peak was found to be increased while CF peak was decreased. This solid sample was then extracted by 90-min sonication and immediately subjected to HPLC analysis (injection within 6 min). CF peak was not observed (Figure 5e; Table 2, number 24). The amount of FA was estimated as 0.788 mg/g. The pH value of this extract was 9.06, which was located in the working pH range of this analytical column (pH values between 1 and 10). After all, this sample powder was extracted for 30, 45, 60, 80 and 100 min, respectively. These extracts were allowed to stand overnight (c.a. 10 hr) at room temperature before HPLC analysis (Table 2, numbers 25-29). CF peak was not observed at all, and the amount of FA was estimated to be 0.805 ± 0.003 mg/g with a RSD of 0.70% (n = 5). These results indicated that (a) FA and its conjugated compounds were readily extractable from the herbal sample; (b) CF was first extracted from sample material and then hydrolyzed into FA in medium; and (c) extended sonication could accelerate hydrolyzing CF into FA. Moreover, the amount of FA extracted with this alkaline medium agreed well with that extracted in water. It indicated that the amount of FA extracted in this alkaline medium could represent the total available content which was responsible for the therapeutic effect of **CA** in medicinal decoctions. Therefore, this solvent could be used to quantify for the amount of total available FA in herbs. In order to drive this conversion to completion, a 100-min extraction was proposed as the optimal sonication using this alkaline medium in subsequent studies.

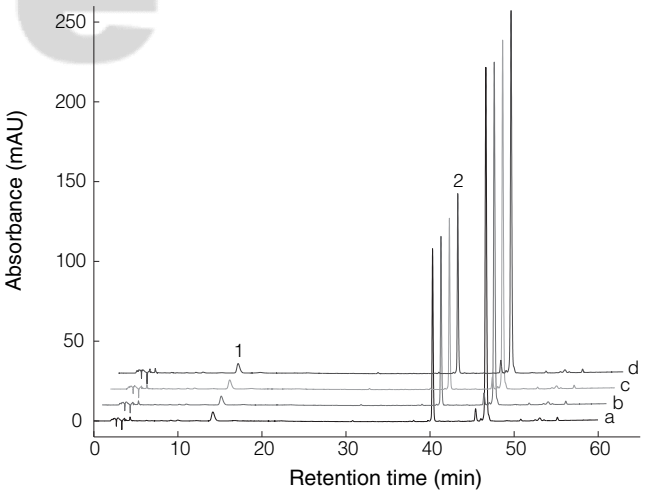

Figure 4. Chromatograms of the root of *Angelica sinensis* (Oliv.) Diels extracted with methanol-formic acid (95:5) by sonication for (a) 30 min; (b) 45 min; (c) 60 min and (d) 90 min, respectively. (1) Ferulic acid; (2) Coniferyl ferulate.

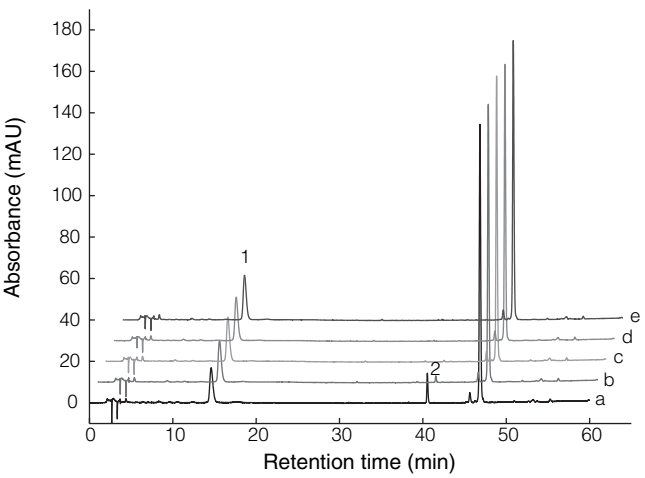

Figure 5. Chromatograms of the root of *Angelica sinensis* (Oliv.) Diels extracted with methanol-2% $NaHCO_3$ in water (95:5) by sonication for 60 min after storage for (a) 5 min; (b) 102 min; (c) 198 min; (d) 390 min and (e) by sonication for 90 min upon standing for 6 min, respectively. (1) Ferulic acid; (2) Coniferyl ferulate.

III. *Validation of Quantitative Analytical Method*

Based on above results, methanol-formic acid (95:5) and methanol-2% $NaHCO_3$ in water (95:5) were chosen as extraction solvents for the assay of free FA and total available FA in **CA** samples, respectively. The measurement wavelength was chosen as 320 nm based on the UV spectra of FA and CF. Under the selected chromatographic condition which was same as that in our previous work[23], FA was fully separated from impurities in the HPLC chromatograms of all 6 types **CA** samples (Figure 6). The validation of new analytical method was examined as follows:

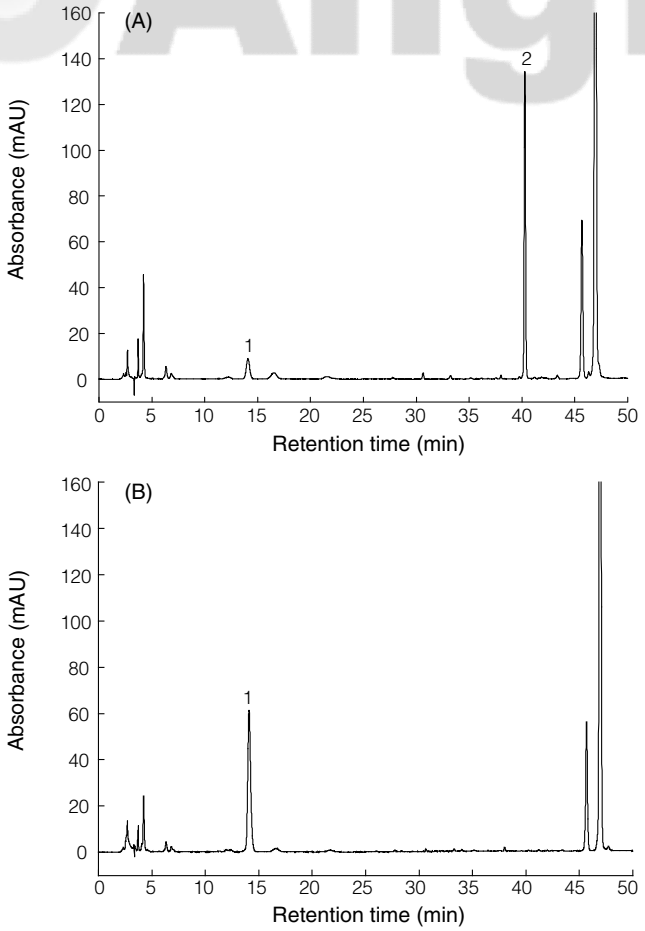

Figure 6. HPLC chromatograms for the assay of (A) free ferulic acid and (B) total available ferulic acid in the whole root of *Angelica sinensis* (Oliv.) Diels. (1) Ferulic acid; (2) Coniferyl ferulate.

(I) Linearity and Calibration Curve: FA was quantified in the tested herbs using an external standard calibration method with a reference marker. Although herbal samples were extracted with neutral, acidic and alkaline solvents in this study, the retention time and UV spectrum of FA were found to be consistent. Therefore, methanol was chosen as the solvent for the preparation of FA standard stock and calibration solutions replacing the acidic and alkaline solvents. The calibration curve was constructed by plotting the peak areas (mAU s) of the analyte versus the concentration (mg/L). It was found that FA was linear over the selected concentration range from 1-25 mg/L with 7 concentration levels. The linear regression equation of the calibration curve was y = 60.256 x – 19.628 with correlation coefficient R^2 = 0.9994 (n = 7). This equation was then employed to calculate the amount of free and total available FA in sample extracts.

(II) Recovery: Comparable amounts of free and total available FA were respectively fortified into the sample powders in six replicated analyses. These spiked samples were extracted and analyzed the amounts of free and total available FA. The recoveries were estimated to be 97.25 ± 4.05% (mean ± RSD, n = 6) for free FA and 98.93 ± 1.47% (n = 6) for total available FA.

(III) Repeatability: six replicates of the solid sample were analyzed and six sample powders were extracted and analyzed the free and total available FA, respectively. The RSD on the contents of free and total available FA in the sample replicates were estimated to be 1.68% and 2.05% (n = 6), respectively.

(IV) Limit of Detection (LOD) and Limit of Quantitation (LOQ): LOD and LOQ were respectively estimated at the signal-to-noise ratios of 3:1 and 10:1. For the assay of free FA, LOD was estimated to be 4.5 μg/g whilst LOQ was found to be 13.8 μg/g. For the assay of total available FA, LOD and LOQ were determined to be 5.8 and 18.5 μg/g, respectively.

IV. *Determination of Free and Total Available Ferulic Acid in 6 Types of **CA** Samples*

In order to investigate availability of the developed method and the amount variation of free FA and total available FA in different types of **CA** samples, 20 **CA** samples of 6 types were collected and quantitatively analyzed. Sample solution and duplicate of each herb were twice injected and analyzed. The means of the contents of free FA and total available FA are shown in Table 1. The peak intensities of FA and CF were noticeably different in the HPLC chromatograms for the assay of free and total available FA. CF peaks were not observed in the chromatogram for assay of total available FA, indicating the developed method was suitable for the analysis of various types of **CA** samples. The amount of total available FA was larger than that of free FA, and their ratios varied remarkably from 1.29 to 8.23 with RSD of 63.7% (n = 20). Also, the average ratio of total available FA to free FA in non-processed samples (whole root, root head and rootlets) (4.30 ± 2.10, n = 11) was larger than that in processed sample (whole root slice, Angelica processed with Chinese yellow wine and charred Angelica) (1.75 ± 0.30, n = 9). It was worth noting that sample form, processing method, water content in herb, storage duration, and humidity in the storage conditions were likely the factors for the observed variations.

CONCLUSIONS

FA commonly co-exists in free and conjugated forms in plants, and some of these conjugated forms are readily converted into the free form during sample extraction resulting in a various amount of FA. Therefore, the extraction condition should be optimized and controllable to obtain an accurate and reproducible result of FA in herbs for assay purpose. Slightly acidic and alkaline solvents were found to be better extraction solvents to develop a rapid analytical method for the assay of FA in natural abundance and total available amount in herbs.

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This study also reveals that total available FA is more abundant than free form and their ratios vary significantly among different types of **CA** samples, and the former is suggested as an appropriate indicator for the quality assessment of herbs. Furthermore, the developed analytical method was accurate and rapid to determine a reliable and comparable FA content in large samples.

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